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CHARACTERIZATION OF PARTIALLY-UNFOLDED INTERMEDIATES IN THE UREA-INDUCED UNFOLDING OF RIBONUCLEASE A

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By

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ABSTRACT

CHARACTERIZATION OF PARTIALLY-FOLDED INTERMEDIATES IN THE UREA-INDUCED UNFOLDING OF RIBONUCLEASE A

by Sangita Seshadri

The stabilizing effect of methanol on the partially-folded intermediate states of ribonuclease A was examined by urea-gradient gel electrophoresis, UV absorbance and fluorescence. Urea-gradient gels were prepared form aqueous and aqueous-methanol solutions at pH 3, 4 and 5. The absorbance (286 nm) and fluorescence (280/305) were measured at 17 ± 1^{0}C. The solvent conditions used were aqueous and aqueous-methanol systems at pH 3, 4, 5 and 6. The electrophoretic patterns observed for the aqueous and 25% methanol systems were consistent with a rapid two-state transition. The 35% and 50% methanol systems suggest a continuous and slightly less cooperative transition. The unfolding transitions observed with the three techniques were not always coincident which indicated the presence of partially-folded intermediates. Transition free energies calculated for absorbance and fluorescence were similar and were less than that obtained from urea-gradient gel data.

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CHAPTER I

INTRODUCTION

1.1 OVERVIEW:

The process by which proteins fold into their complex three-dimensional structure has been the subject of debate and investigation. The problems involved in determining the folding pathway of a protein, and of understanding the folding mechanisms in terms of the energetics and kinetics of the intermediates, has its origins in work that goes back at least half a century.

Protein folding can be defined as the process which includes all the events occuring from translation (biosynthesis of the amino acid sequence) to the formation of the functional structure of a protein molecule. Folding of a protein appears generally to be a self assembly process, in that all the information required is present in the amino acid sequence, and that it occurs spontaneously under the appropriate conditions. Proteins that do not refold in vitro are generally assumed to have undergone

some interfering covalent modification or to have lost some cofactor required for the folded state.

Considering the vast amount of crystallographic data obtained for over 100 structures of proteins, the stage would appear to be set to determine the pathway of folding that should eventually reveal the mechanism of folding and help in elucidating the "code" by which the amino acid sequence of a protein specifies its three dimensional structure (1). Knowing the folding code will not only aid in engineering changes in protein structure using recombinant DNA techniques, but also provide a tool to specify hypothetical functions for given amino acid sequences deduced from DNA sequence analysis. Furthermore, with the knowledge of the folding code, it will be possible to design and tailor proteins with specific properties using recombinant DNA techniques.

In 1932 Northrup (2) reported that the thermal denaturation of trypsin at pH 2 follows a reversible transition curve. In the early 1950's, Kauzman and his coworkers (3) were able to demonstrate that protein denaturation is the unfolding of the ordered, three

dimensional structure of a protein. Refolding was demonstrated shortly thereafter by Anfinsen and coworkers (4). In the early 1960's Kendrew and co-workers (5) solved the X-ray structure of myoglobin and Phillips and co-workers (6) followed with the lysozyme structure. By the mid 60's the tools needed for the work on the pathway and the mechanism of folding were at hand.

Since the early experiments of Anfinsen, it has been recognized that protein folding is a spontaneous event and proteins are observed to fold on time scales of 10^{-1} to 10^{-3} sec. The time scale leads to the inescapable conclusion that proteins do not fold by simply sampling randomly all possible conformations until that with the lowest free energy is encountered. Instead, folding must proceed via locally folded intermediates that function as sites for cooperative growth (7).

1.2 MODELS FOR PROTEIN FOLDING:

A large number of mechanistic models for protein folding have been proposed. These models are of two kinds: structural and kinetic (8). Since the structural data on

the intermediates are only starting to be available, both classes of models still consist of hypotheses about the folding process. Present structural models are based mainly on the X-ray crystallographic structures and the NMR data of native proteins. The aim of the structural model is to give the actual structures of the intermediates as well as their order on the pathway, without being too specific about the factors that control the rate of folding.
The aim of the kinetic model is to indicate the dominant intermediates and determine the factors that control the rate of folding without being too specific about the structures of the intermediates.

1.2.1 Structural Models

The increasing availability of X-ray crystallographic structures for proteins has stimulated the proposal of many structural models for protein folding. Some models postulate that folding begins by formation of a "primitive" H-bonded structure that breaks down to generate the observed structure. Ptitsyn & Finkelstein (8) postulated a model for all beta proteins, according to which the β-proteins have long two-stranded antiparallel

β-structure with a central hairpin loop. The Greek key (fig.1) patterns of connections between β-strands then result from breaking this hairpin helix into shorter segments by opening unpaired loops. The particular "swirl" of the greek key resulted from the right-handed twist of the β-sheet.

1.2.2 Kinetic Models:

A number of kinetic models have been proposed by different research groups. Four major themes for the folding of proteins are discussed below:

1.2.2.1 Baised Random Search: The possibility that proteins might fold by a purely random search of all possible conformations was considered by Levinthal (10) and then dismissed, because the time required for folding would be impossibly long [10 years for a chain of 100 residues]. However, it is possible that biased random searchess could occur in reasonable time.

By means of computer simulated folding, it was shown that the number of possible chain conformations could be reduced if only self-avoiding (sterically possible) conformations are allowed (11). Levitt also found that

these self-avoiding conformations are fairly compact, and that rapid folding might begin whenever the conformation of the backbone of the unfolded chain is sufficiently similar to that of the native protein. On this basis it was suggested that a biased random search could play an important role in the early stages of folding.

1.2.2.2. Nucleation-Growth: The nucleation-growth model assumes that the folding reaction proceeds rapidly as soon as a nucleus is provided (as in crystallization of a supercooled liquid after seeding with a crystal, or as in a helix formation). In addition, the nucleated molecule is not observable as a populated species either because folding occurs rapidly after nucleation or because the nucleus is unstable by itself and breaks down if it is not stabilized by further folding (12). Furthermore, folding intermediates are not populated, because folding is too fast once it starts, and the rate of folding is determined by the nucleation reaction. Since populated kinetic intermediates have been demonstrated in the folding of several proteins, it appears that at least in these cases, protein folding is not a nucleation-limited reaction.

1.2.2.3. Diffusion-Collision-Adhesion: The diffusion-collision model considers the protein to be divided into several parts (microdomains) (13), each short enough for conformational alternatives to be searched through rapidly. Each of the microdoamins can be described as being in equilibrium between the native and the unfolded random-coil structure. A step-wise folding mechanism is likely. For example, two units coalescing to form a slightly more stable entity which in turn collides with a third entity, and so on. The diffusion collision mechanism would be most effective if the individual segments (microdomains) were relatively unlikely to bend significantly; that is they can be regarded as rigid rods in the diffusion process, so as to minimise entanglement problems. Once the segments have diffused together, the balance between the hydrophobic attractions and the van der Waals repulsion will determine whether or not coalescence could occur in a given collision.

1.2.2.4. Sequential folding: In the sequential folding model, folding occurs in a unique and definite sequence of steps. Intermediates may be populated under suitable

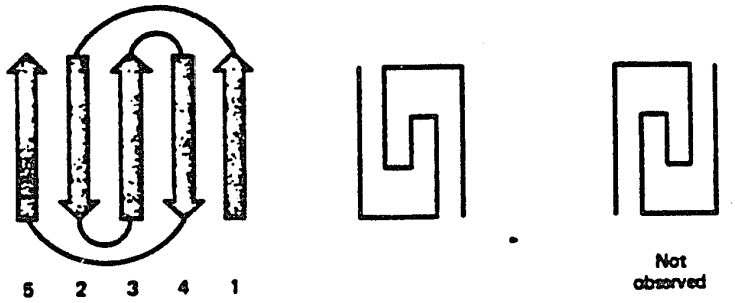

Figure 1. Greek Key Structure.

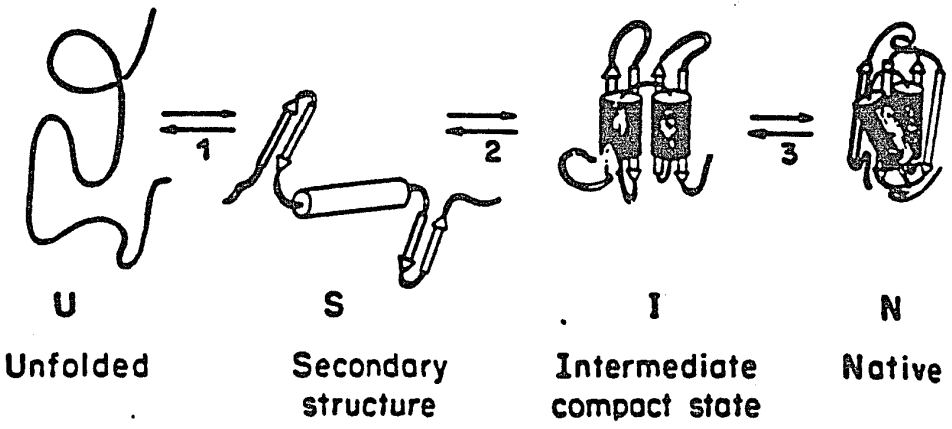

Figure 2. Framework Model.

conditions of folding.

Two working models are currently popular (8). The first one is the `framework model' (14, 15, 16a & b), (fig 2), in which the hydrogen bonded secondary structures are formed early in the folding process, which then coalesce together to form an intermediate globular structure. This intermediate structure rearranges itself to the final specific native tertiary structure. The essential feature of this model is that different states are stabilized by different interactions: (1) secondary structures by peptide hydrogen bonds, (2) intermediate globular structures by hydrophobic interctions, and (3) specific tertiary structures by van der Waals interactions.

The second is the `modular assembly model' (17), in which essentially complete folding of any part of the protein occurs at one time, although different parts of the protein fold at different times.

These two models may be combined; a protein may consist of quasi-independent domains or of sub-domains that may fold at different times, as in the modular assembly, but each may fold according to the framework model (17).

1.3 GENERAL APPROACH TO STUDY PROTEIN FOLDING:

The general approach for studying protein folding has been to study either the equilibrium transition or the kinetics of unfolding or refolding.

1.3.1 EQUILIBRIUM STUDIES

It is generally accepted that the folding transition of typical, small, globular proteins are highly cooperative and that the two state approximation is a good working model for equilibrium studies.

The two state approximation can be applied if no intermediates are present at equilibrium (fig.3a). These studies can then evaluate the thermodynamic differences between the native and the unfolded forms, or compare native with modified proteins.

The two state approximation does not apply if at equilibrium there exists low levels of structured folding intermediates or high levels of intermediates which are nearly indistinguishable from N (native) or U (unfolded) states (fig.3b). Sensitive methods can detect these intermediates and prove useful in elucidating the mechanism of folding.

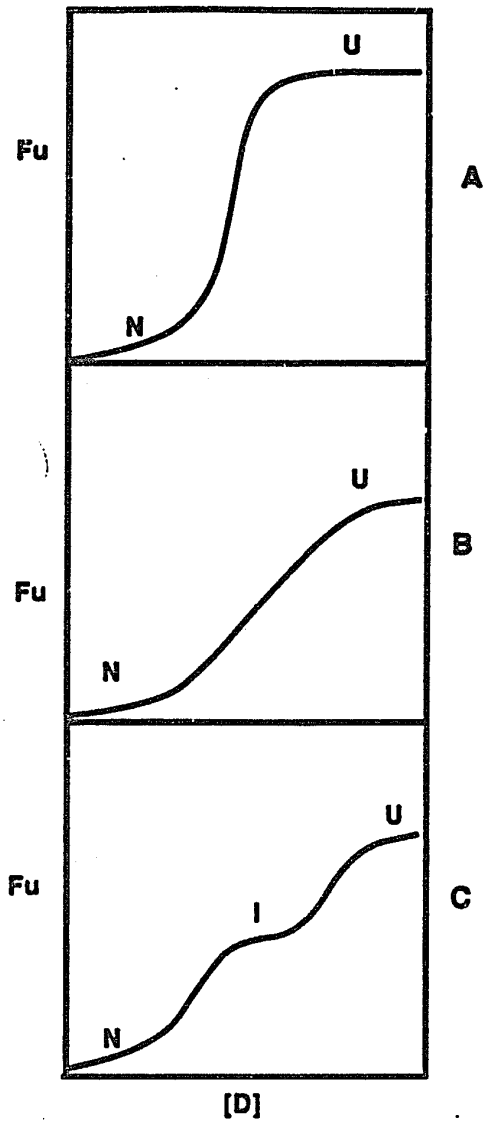

Figure 3. Equilibrium Transition
A) Cooperative transition B) Non-cooperative transition C) Multiple uncoupled equilibria

On the other hand, one can have multiple uncoupled equilibria (fig. 3c), where the native state is in equilibrium with the intermediate which is in equilibrium with the unfolded state.

1.3.2 KINETIC STUDIES

In order to examine kinetic intermediates, the folding transition should be reversible and the native protein should be recovered in 100% yield upon refolding. Aggregation reactions are the most common cause of irreversibility, and they interfere with the observation of intrinsic folding intermediates. In a reversible reaction the folding pathway can be studied from both ends, i.e. in the folding or the unfolding direction.

The study of kinetic intermediates took a new turn with the finding (19) that unfolded RNase A contains both fast-folding (Uf) and slow-folding (Us) forms. Uf and Us have been shown to co-exist in slow equilibrium (20). A plausible explanation for the presence of the Uf and Us species in the unfolded protein was given by the proline model (first proposed by Brandts and coworkers); Uf and Us differ in the cis-trans configuration of the X proline

peptide bonds. It was shown that Uf represented 20-25% of the unfolded molecules, and Us represented the remaining 75-80%. The slower folding is thought to arise from the isomerization of the thermodynamically stable form in the unfolded state to the cis configuration. Isomerization would apply only to Pro-92 and -114 which are in the cis configuration in the native state.

The Us state has subsequently been divided into two subclasses, UsI, the minor pathway (16% of total), and UsII, the major pathway (64% of the total). The configuration about Pro-93 defines the folding of UsI (21). The differences between these subclasses are the subject of much controversy.

1.4 DETECTION AND CHARACTERIZATION OF INTERMEDIATES IN AN UREA INDUCED EQUILIBRIUM UNFOLDING PATHWAY:

In order to understand how proteins fold, one must know the factors that control the folding pathway and thereby define the native structure. The approach chosen to accomplish this involves the characterization of native, unfolded, and partially folded structures in as precise a

manner as possible. Comparison of a number of such structures should allow one to deduce the folding pathway. Ideally one would like to obtain crystallographic data for all such structures. Presently, only the native states of the proteins lend themselves to such high resolution structural characterization. NMR spectra also contain a wealth of structure information. They help in determining the structure of the native, intermediate and the unfolded states, although in a less precise manner.

The main aim of this project is to determine the experimental conditions necessary to stabilize the partially-folded intermediates in the folding of RNase and characterize these structures in as precise manner as possible. Determination of the conditions and characterization of the intermediates was done by monitoring the absorbance and fluorescence of the protein. Information regarding the changes in the conformation of the protein occurring under different experimental conditions was obtained by monitoring the absorbance and fluorescence. Urea gradient gel electrophoresis was performed to get an idea about the changes in the overall

hydrodynamic radius and the exposure of charged groups. The determination of such conditions yields additional information regarding the conformational stability of the protein, ΔG of the protein, and the presence of partially-folded intermediates.

The experimental conditions involved systems of different pH (3-6), urea concentration in the range 0-8M and different solvent systems (aqueous, 25%, 35% and 50% methanol).

1.4.1 Classes of cosolvents and protein-solvent interaction:

One can distinguish two major classes of cosolvents, based on qualitative differences in their effects on protein stability (22). Polyols increase the stability of the protein, whereas monohydric alcohols decrease the stability of the native protein. The decreased stabilizing effect of monohydric alcohols is due to the unfavourable interaction between the solvent and the protein surface. This is due to the non-polar character of the monohydric alcohols. Hence, a protein with highly polar surfaces, such as ribonuclease A will be destabilized by monohydric

alcohols. In case of polyols the apparent stabilization of the native state actually results from greater destabilization of the unfolded state due to its larger surface area. An aqueous-methanol solvent system was chosen to stabilize the partially-folded intermediates over the native and unfolded states. Solvent systems with hydrophobic character are known to destabilize hydrophobic interactions in proteins and stabilize the hydrogen bonding in the secondary structures (23). This helps in stabilizing the partially-folded states relative to the unfolded and the native states. The hydrophobic interaction between the organic co-solvent and the protein causes the destabilization of intra-protein hydrophobic interactions. Stabilization of hydrogen bonding is believed to be due to the decrease in favourable water-protein hydrogen bonding by the reduction of water concentration about the protein molecules.

In the presence of methanol, the partially-folded state is stable relative to the unfolded and the native state. Urea destabilizes the native state and stabilizes the unfolded, whereas methanol destabilizes the native and the unfolded states and stabilizes the partially folded

states. Methanol stabilizes the secondary structures, mainly the alpha helix. Thus, a combination of urea and methanol can enable in populating and characterizing the intermediates.

The protein chosen for studying the protein folding pathway was bovine pancreatic Ribonuclease A (RNase A). RNase A, is a small, single domain protein. This protein has been well studied and offers a number of advantages (24): (1) the crystal structure is known; (2) its folding is characterized by multiple intermediate states; (3) the solubility in water and aqueous-methanol solvents is quite high; (4) it has a low tendency to aggregate even in the unfolded state; (5) unfolding and subsequent refolding is reversible; and (6) aqueous-methanol solvents have no adverse effects on the catalytic activity.

In order to determine the the folding pathway it is necessary to have structural information regarding the native, intermediates, and the unfolded state and their relative energy states. In addition, the energy barriers between the states should also be known. By analyzing the denaturant curves of proteins it is possible to obtain an estimate of the conformational stability, of the protein,

i.e., how much more stable the globular, native conformation of the protein is in comparison to the unfolded, denatured conformation.

1.4.2 Spectroscopic probes to monitor unfolding:

Fluorescence and UV absorbance measure the exposure of the aromatic residues to the bulk solvent and give a crude measure of the tertiary structure of the protein.

1.4.2.1 Absorbance:

Absorbance of UV light by peptide bonds and non-aromatic residues is not very sensitive to conformation or environment as compared to the absorbance by the aromatic rings of phenylalanine, tyrosine, and tryptophan. The degrees of solvent exposure measured from the absorbance spectrum of the protein and from its perturbation by solvents are complementary. Changes in the environment such as from a polar environment to a non-polar environment have a large effect on the energy difference between electronic states, leading to a corresponding change in the absorption wavelengths. In the case of RNase A, three tyrosines are exposed and three are buried within

the nonpolar regions of the protein. The predominant transitions in the 250 to 300 nm region are π^*. When the protein unfolds, the buried tyrosines are exposed and the environment around them changes from a nonpolar to a polar environment. This change in the environment will cause a decrease in the energy separation between π and π^* levels and cause a red shift in the absorption spectrum.

1.4.2.2 Fluorescence:

Fluorescence by the aromatic groups is much more sensitive to the local environment. An increase in the quantum yield has been observed as the protein unfolds. In case of RNase A, the fluorescence of the tyrosine is monitored. When the protein is in the native state the fluorescence of tyrosines is quenched and this results in the low yield of fluorescence. But as the protein unfolds, quenching decreases and the quantum yield increases. Quenching is due to the partial abstraction of the phenolic proton in the excited state (by a proton abstractor such as -COO or NH) (25) as is the case for the strongly hydrogen bonded tyrosine residues. Abstraction of the proton results in a significantly reduced quantum yeild.

1.4.3 Urea Gradient Gel Electrophoresis:

Urea gradient gel electrophoresis measures primarily the compactness of the protein molecule as reflected in its rate of migration through concentrated polyacrylamide gels, although the net charge will also affect the electrophoretic mobility. The electrophoretic mobility of a protein through polyacrylamide gel is directly proportional to the number of exposed charges and inversely proportional to the hydrodynamic radius. The unfolding of the protein caused by a denaturant such as urea increases the hydrodynamic radius of the protein and the number of exposed charges. Consequently, the unfolded and the partially-folded proteins have a reduced mobility as compared to the native state. Urea gradient gel electrophoresis (26-28) exploits this phenomenon. From a single experiment one can obtain information regarding the entire urea concentration mediated transition and regarding the tertiary structure of the protein.

Thus, structural characterization of partially-folded intermediates will provide an insight into the mechanism by which proteins fold and help decipher the code for protein folding.

CHAPTER II

MATERIALS AND METHODS

2.1 MATERIALS:

Bovine Pancreatic Ribonuclease A, mercaptoethanol, sephadex SPC-25 (40-120u) and G-25 were purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoresis grade Tris base, dithiothreatol and sodium iodoacetate were purchased from Fisher Scientific (N.J.). Ultrapure urea was purchased from Schwartz-Mann Biotech.

2.2 METHODS:

2.2.1 Purification of Ribonuclease A:

Ribonuclease A was purified (29) further on a Sephadex SPC-25 column (1.5 x 25 cms, 0.13M phosphate buffer, pH 6.5). In a typical purification, a 200 mg sample was applied to the column in 1-2 ml of the eluting buffer. This procedure facilitates the separation of RNase S and aggregated material from RNase A. The sample was dialysed to remove the residual phosphate, then lyophilized and

stored at -20 ^{0}C.

2.2.2 Reduction and Carboxymethylation of RNase A

The reduction and carboxymethylation of RNase A was accomplished according to the procedure of McWherter et al. (30).

A 20 mg sample of RNase A was dissolved in 1 ml of the reaction buffer (0.5 M Tris, 6M guanidine-HCl, and 2.7 mM EDTA). It was then heated at 65^{0}C for 10 min, cooled to 50^{0}C and then deoxygenated by bubbling nitrogen through the solution for about 30 minutes. After the removal of oxygen, 49 mg of Dithiothreatol was added and the reduction was allowed to proceed under nitrogen atmosphere for 4 hours. The solution was cooled to room temperature and the reduced RNase A was carboxymethylated by addition of 0.14 gms of sodium iodoacetate dissolved in 0.5 ml of the reaction buffer. After 20 minutes, the remaining iodoacetate was destroyed by the addition of 2 ml of 2-mercaptoethanol.

The reaction mixture was purified on a G-25 column equilibrated in 0.1 M acetic acid, dialysed and finally lyophilized. The extent of carboxymethylation was checked by performing the DTNB (Dithio bis-nitro benzene) (31)

test. This allows detection of remaining free sulfhydryl groups.

2.2.3 Urea Gradient Gel Electrophoresis

Urea gradient gel electrophoresis was performed to measure the compactness of the protein as reflected in its rate of migration through a polyacrylamide gel.

Urea gradient gels (16 x 13 x 0.5 cms) were made by photopolymerizing the acrylamide solutions in an in-house manufactured slab gel casting apparatus in which a gradient of urea had been formed by mixing two acrylamide solutions with the two limiting concentrations of urea (usually 0M and 8M).

A small amount of the low urea concentration solution, sufficient to fill the top 1 cm of the gel, is first pumped into the apparatus. This is followed by the linear gradient of urea. This gradient is generated by adding, with rapid mixing, the high urea concentration solution to a predetermined volume of low urea concentration solution. The natural density of urea solution helps in stabilizing the gradient. After the

gradient is formed, more of the high urea concentration solution is pumped in, so that the gel is finally composed of a 1 cm length of low urea concentration solution, the gradient, and 1 cm length of the high urea concentration solution. Since oxygen inhibits polymerization of acrylamide, the gels were over-layered with isobutanol to prevent contact with air.

All gels contained an acrylamide gradient to compensate for the increased viscosity of urea (26). The reverse acrylamide gradient was determined empirically for each buffer condition (Table 1).

The gels were photopolymerized with the help of two 5 watt "cool white" fluorescent tubes. Since occurrence of polymerization has to be prevented until the gradient is formed, the technique of photopolymerization was preferred to that of regular polymerization.

The gels contained 5 mg/liter riboflavin and 0.12% (v/v) N, N ,N′ ,N′ -tetramethylethylenediamine (TEMED) as polymerization catalysts. These catalysts alone will cause the polymerization of the gels of relatively high acrylamide concentration (>10%) and low pH (<5) in about 15

minutes.

The linearity of the urea gradient was confirmed by optical scanning at 310 nm of several gels along three different regions of each gel. Scanning (at 310 nm) along the gradient revealed a linear increase in absorbance (due to scattering) with increasing urea concentration. Scanning perpendicular to the gradient revealed no such change. The gradient forming methodology was also checked by including a dye in one of the acrylamide solutions, pouring a gradient gel and then measuring the dye concentration by densitometry.

After photopolymerization, the urea slab gels were inserted into the electrophoresis apparatus so that the urea gradient is perpendicular to the direction of electrophoresis.

The gels were pre-electrophoresed for 1 hour at 50-60 V, 10 mA, and constant power. Then, 50 uls of protein solution (2 to 3 mgs /ml of 0.05 M tris buffer and 10% glycerol) was loaded onto the gel. Glycerol was included to prevent thermal mixing.

Electrophoresis was performed at 17 + 1 ^{0}C, 30 watts

constant power, and a current of 50 mA for 2.5 to 5 hours depending on the conditions. A tracking dye (pyronin G) was included to monitor the progress of electrophoresis and sample application. The gels were fixed in 50% TCA and stained with 0.1% Coomasie Brilliant Blue overnight.

Urea gradient gel electrophoresis was performed in aqueous and aqueous-methanol solvent systems at pH 3, 4 and 5 (Table 1). These buffers were prepared on a v/v basis (for example, 35:65 methanol-aqueous buffer). The upper and the lower electrophoretic chambers were filled with the respective aqueous buffer.

2.2.4 Unfolding monitored by Absorbance and Fluorescence:

Absorbance measurements were carried out at 286 nm on a Hewlett-Packard Diode Array Spectrophotometer and fluorescence measurements were carried out with a Perkin-Elmer LS-3 spectrofluorometer with the excitation set at 280 nm and the emission at 305 nm at 17 + 1 ^{0}C. An insulated, thermostated brass-block cell holder was used to maintain the temperature in the sample cuvette. Cooling was provided by a Forma Scientific temperature control bath

Table 1.
Solvent Systems

pH	Buffer System	Conditions	Acrylamide Reverse Gradient
3	0.05 M Tris-formate	aqueous 25 % methanol 35 % methanol 50 % methanol	15 % - 12 % 15 % - 12 % 15 % - 10.5 % 15 % - 10.5 %
4	0.05 M Tris-formate	aqueous 25 % methanol 35 % methanol 50 % methanol	15 % - 14 % 15 % - 14 % 15 % - 13.5 % 15 % - 13.5 %
5	0.05 M Tris-formate	aqueous 25 % methanol 35 % methanol 50 % methanol	15 % - 12 % 15 % - 11.25 % 15 % - 11.25 % 15 % - 11.25 %
6	0.05 M Tris-formate	aqueous 25 % methanol 35 % methanol 50 % methanol	

(model # 2095).

In a typical experiment, urea stock (8.39 M) was prepared in the buffer (see Table 1) from which other intermediate dilutions were prepared so as to obtain a complete set of dilutions from 0-8 M urea.

A protein stock solution of 10 mg/ml was prepared in the same buffer. 100 uls of the protein sample was added to 2 ml of urea solution and incubated for one hour (in order to obtain a stable baseline), after which the absorbance and fluorescence were measured.

The protein concentrations were determined by adding 100 uls of the protein sample to 2 ml of the buffer and measuring the absorbance at 278 nm (molar absorptivity of RNase A = 9800).

The same procedure was carried out at various other conditions (see Table 1).

2.2.5 Denaturation Curve Analysis:

The data from all the experiments were analysed on a spread-sheet program, EXCEL, as indicated below:

Absorbance, fluorescence and Rf values (in the case

of urea gel electrophoresis) were used as the parameters (y) to follow the urea induced unfolding of RNase A. RNase A has been found to approach a two-state mechanism (32), in which only the native N and the unfolded U are present at significant concentrations in the transition region. The values for the y characteristic of the native state, yN, and of the unfolded state, yU, were obtained in the transition region by extrapolation from the linear portions of the denaturation curve at low and high denaturant concentrations (fig. 4). These linear changes result from solvent effects on the properties of the native and denatured states.

For a two state mechanism, fN + fU = 1, and y = yN x fN + yU x fU where fN and fU represent the fraction of the protein present in the native and the unfolded states (fig. 4). Combining these equations,

$$fU = (y - yN)/(yU - yN)$$

and

$$fN = (yU - y)/(yU - yN)$$

The relationship between the change in free energy (ΔG) and equilibrium constant (Keq) is given by:

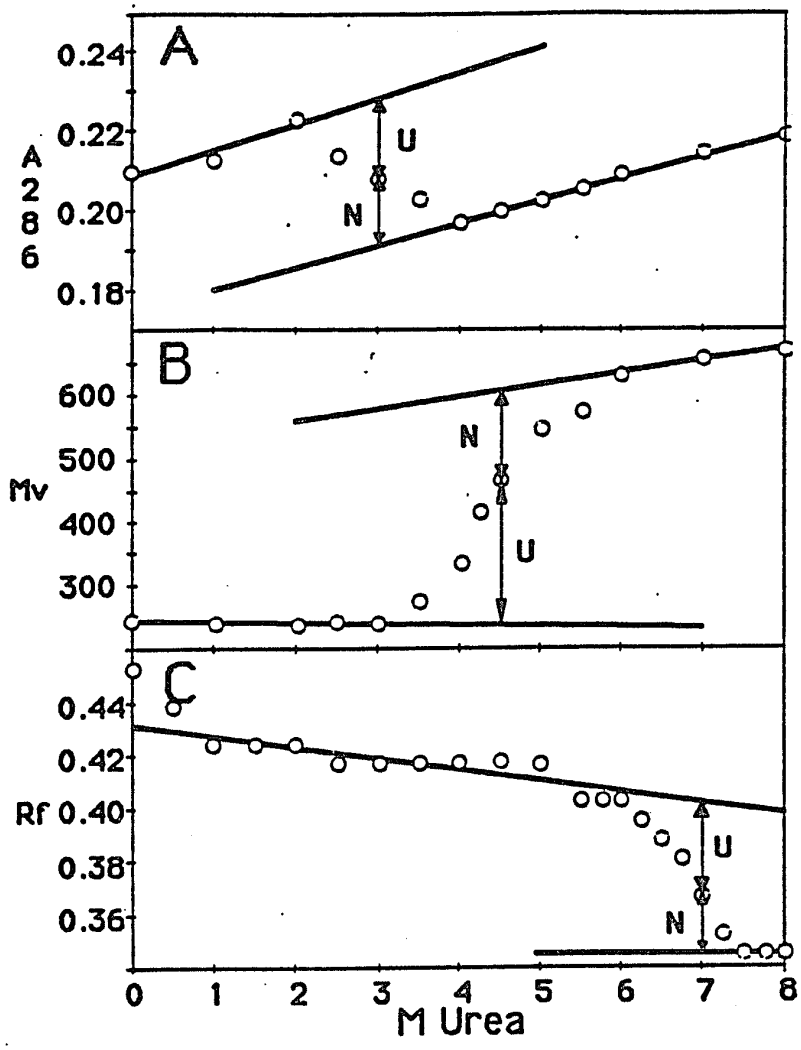

Figure 4. Data Analysis A) Absorbance B) Fluorescence C) Urea-Gradient Gel

$$\Delta G = -RT \ln(Keq) = -RT \ln [product] / [reactant]$$

$$i.e. \Delta G = -RT \ln fU / fN$$

Change in free energy can be calculated using

$$\Delta G = -RT \ln (fU) / (1-fU)$$

The intercept (ΔG_{app}) values were calculated by extrapolating a linear least squares analysis of a minimum of two sets of Fu data data to OM urea.

Standard deviation (33) were computed for each of the analysis by the following relation:

For an expression; $y = mx + b$

Standard deviation for the least square analysis is

$$\sigma^2 b = \frac{\sigma^2 \Sigma x^2}{\Delta}$$

where, $\sigma^2 = \frac{1}{N} \Sigma (mx_i + b - y_i)^2$ and $\Delta = N \Sigma x_i^2 - (\Sigma x_i)^2$

CHAPTER III

RESULTS & DISCUSSION

3.1 FLUORESCENCE:

The urea induced unfolding of RNase A was monitored by the change in fluorescence at 280 nm excitation and 305 nm emission for each of the solvent systems given in Table 1. An increase in the fluorescence is observed as the protein unfolds (figure 5). This is due to the decrease in the quenching of tyrosines. The plots of the fraction of unfolded protein present and the change in free energy of transition, both as a function of urea concentration, are shown in figures 6 and 7.

3.1.1 Effect of Methanol on the urea induced unfolding transition:

In general, increasing the methanol concentration resulted in a decrease in the Cm (the urea concentration at which half the protein is in the unfolded state) at a given pH (fig. 8). This was more pronounced at higher pH. In

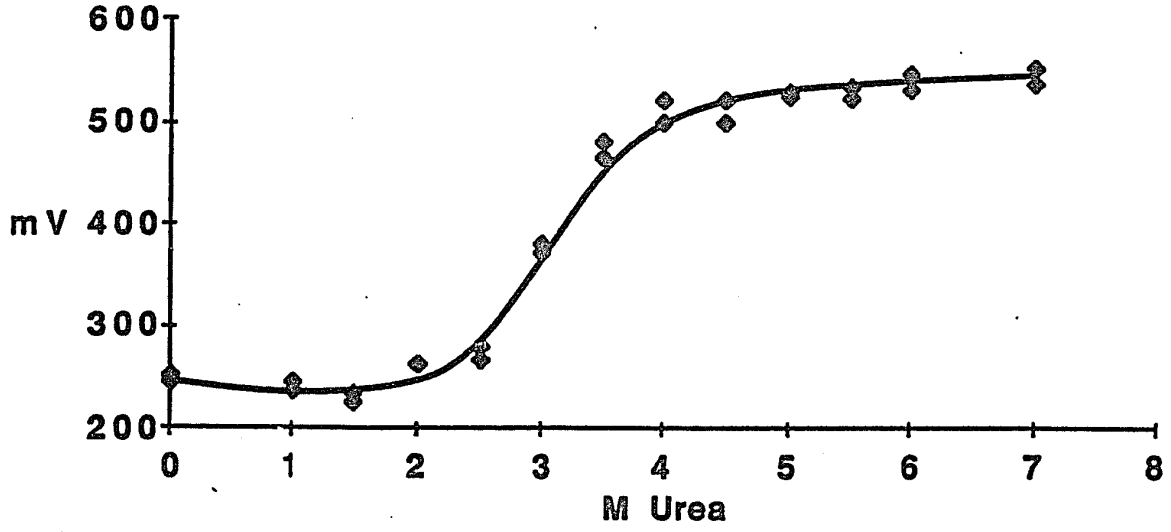

Figure 5. Fluorescence (280/305 nm) of RNase as a function of urea concentration at pH 4.0 (0.05 M tris/acetate) and 17 $\pm^{0}$C

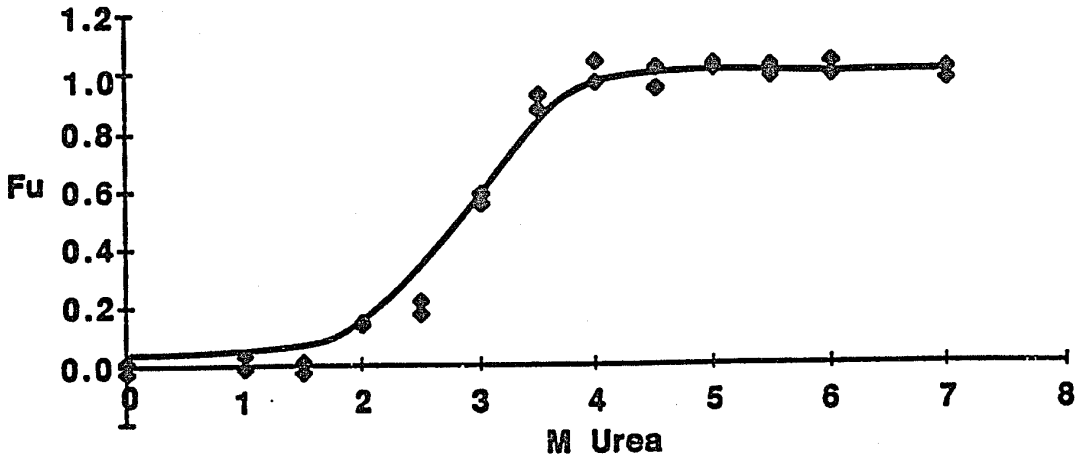

Figure 6. Fraction unfolded of RNase as a function of urea concentration at pH 4.0 and 17 $\pm^{0}$C.

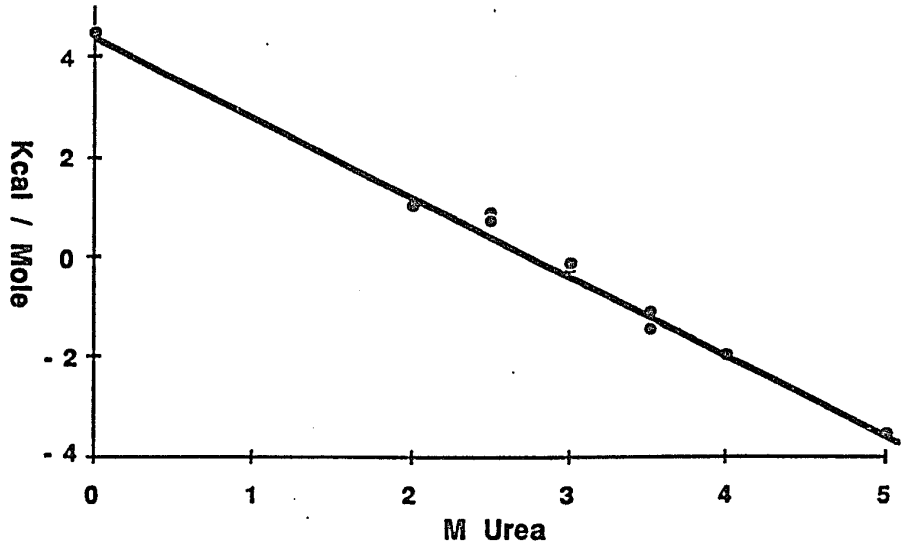

Figure 7. Change in free energy of transition as a function of urea concentration at pH 4.0 and 17 $\pm^{0}$C.

addition, it was found that the transition was less cooperative and covered a wider range of urea concentrations at higher methanol concentrations. This decreased cooperativity may be indicative of populated intermediates.

Specific trends are outlined below:

At pH 3, an increase in the methanol concentration resulted in a small decrease in the Cm. At pH 4, the transitions observed under aqueous and 25% methanol were coincident. Further increase in the methanol concentration resulted in a decrease in the Cm. At pH 5, the transitions were found to be variable, with a slight increase in the Cm with increase in the methanol concentration through 35% methanol and a decrease between 35% and 50%. At pH 6, a decrease in Cm was observed as the % methanol was increased from 0 to 25% after which the Cm was independent of the methanol concentration.

At any given pH, the change in $\Delta G_{apparent}$ with respect to methanol concentration was variable (figure 9). At pH 3, 4 and 5, the change in free energy was maximum under 25% methanol after which a decrease in the free

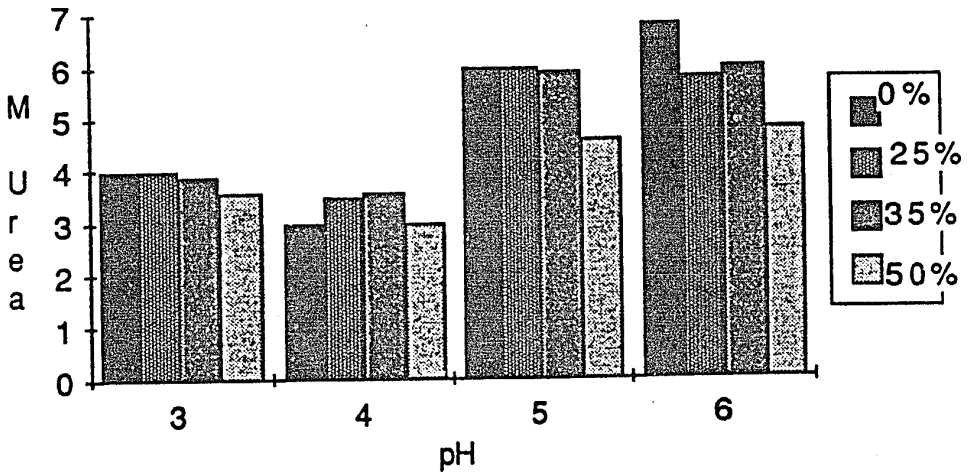

Figure 8. Cm as a function of pH at various methanol concentrations.

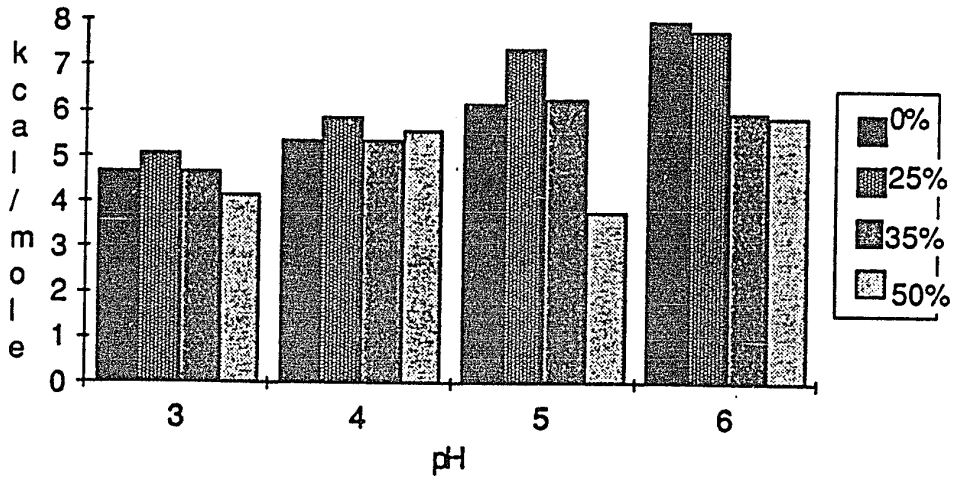

Figure 9. Change in Free energy as a function of pH at various methanol concentrations.

energy was observed. At pH 6, a sharp decrease in the change in free energy was observed with an increase in methanol concentration from 0 to 35%.

3.1.2 Effect of pH on the fluorescence observed during the urea-induced unfolding transition:

In general, at any given methanol concentration, an increase in the Cm was observed as the pH was raised from 3 to 6 (fig. 10). In addition, the transition became more cooperative with an increase in pH.

The only anomaly was observed under 50% aqueous-methanol conditions, where a decrease in the Cm was observed as the pH was increased from 3 to 4, after which an increase in pH resulted in an increase in the Cm. The change in pH from 4 to 5 showed a pronounced increase in the Cm as compared to the change in Cm observed with an increase in pH from 3 to 4 or from 5 to 6. This increase in Cm is indicative of an increase in the stability of the protein at high pH. At higher pH i.e. at pH 5 and 6, the protein is fairly stable and, hence, is not altered by small increments in the methanol concentration. The change

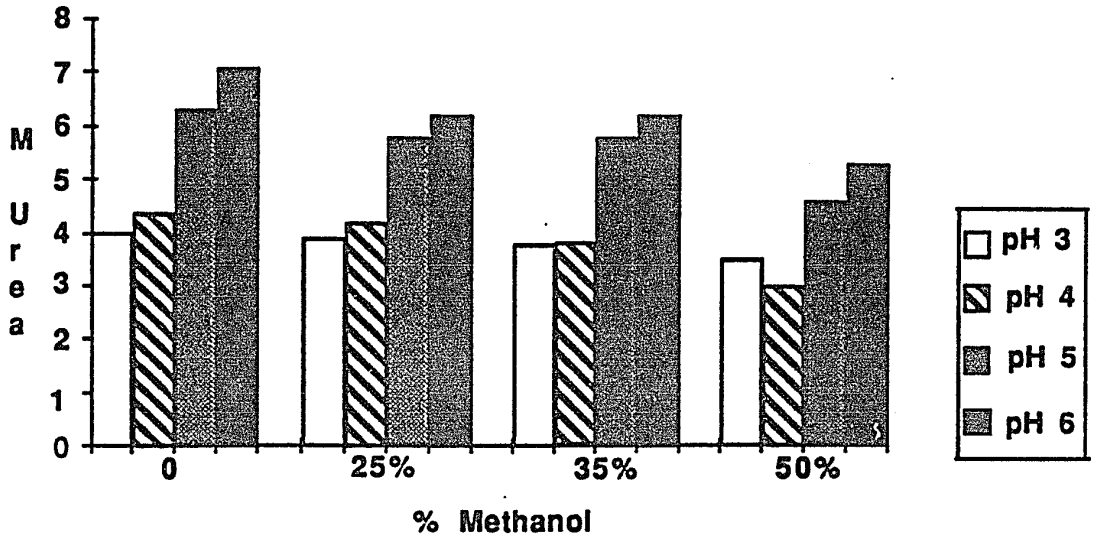

Figure 10. Cm as a function of methanol concentration at pH 3, 4, 5 and 6.

in Cm observed as the pH was increased from 4 to 5 was more than the change observed when the pH was increased from 3 to 4 or 5 to 6. This is probably due to the "charged-group effect"; as at pH 3 and 4, the carboxyl groups are not completely ionized, whereas at pH 5, 90% of these would have been ionized.

At any given methanol concentration, an increase in pH from 3 to 6 resulted in an increase in the free energy change (figure 11). The change in the apparent ΔG value decreases with increasing methanol concentration.

3.2 ABSORBANCE:

The urea-induced transition was determined for each solvent system (Table 1) by monitoring the change in absorbance at 286 nm. At this wavelength, the degree of exposure or burial of tyrosine residues was measured.

As the protein is subjected to increasing concentrations of urea (figure 12), a small linear increase in absorbance is observed from 0M to 2M urea. This can be attributed to the solvent perturbation effect of urea on the electronic absorption of exposed tyrosines in the

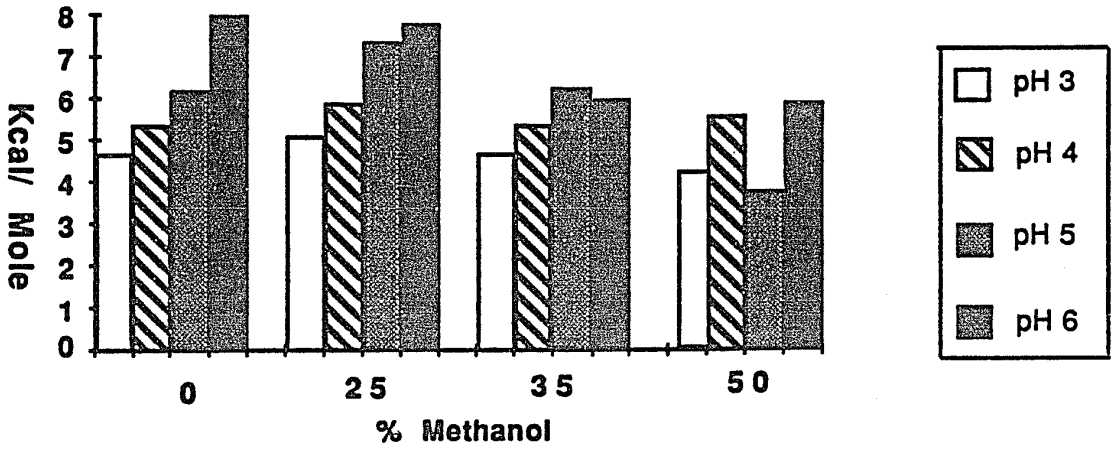

Figure 11. Change in Free-energy as a function of the methanol concentration at pH 3, 4, 5 and 6.

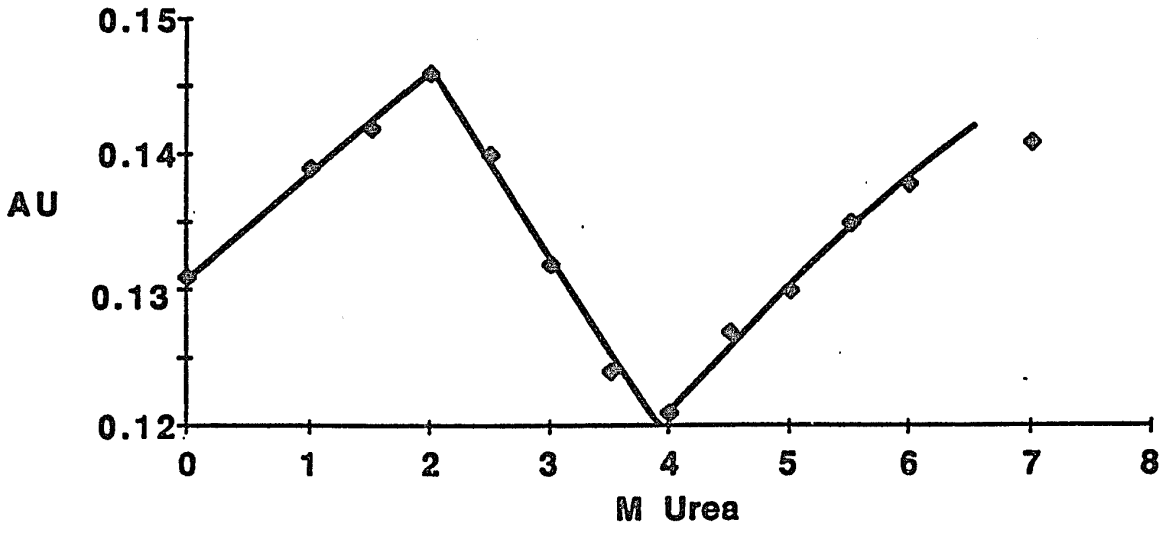

Figure 12. Absorbance of RNase at 286 nm as a function of urea concentration at pH 4.0 (Tris/acetate) and 17 ±^{0}C.

native conformation. Between urea concentrations of 2M and 6M, the large decrease in the absorbance observed shows that the protein undergoes an unfolding transition in this range. Above 6M, where the conformation is relatively stable, the linear increase in the absorbance is again due to the solvent perturbation of the exposed tyrosines in the unfolded conformation by increasing concentrations of urea. Plots of the fraction of unfolded protein present and the change in free energy of transition as a function of urea concentration are shown in figure 13 and 14.

3.2.1 Effect of methanol concentration on absorbance during the urea-induced unfolding transition:

The change in Cm with increasing methanol is variable at any given pH (figure 15).

At pH 4, the transitions under aqueous and 50% methanol conditions are similar and the intermediate concentrations serve to increase the mid-point of the transition and lower the relative cooperativity of the transition.

With one exception, pH 5 aqueous, the Cm and ΔG

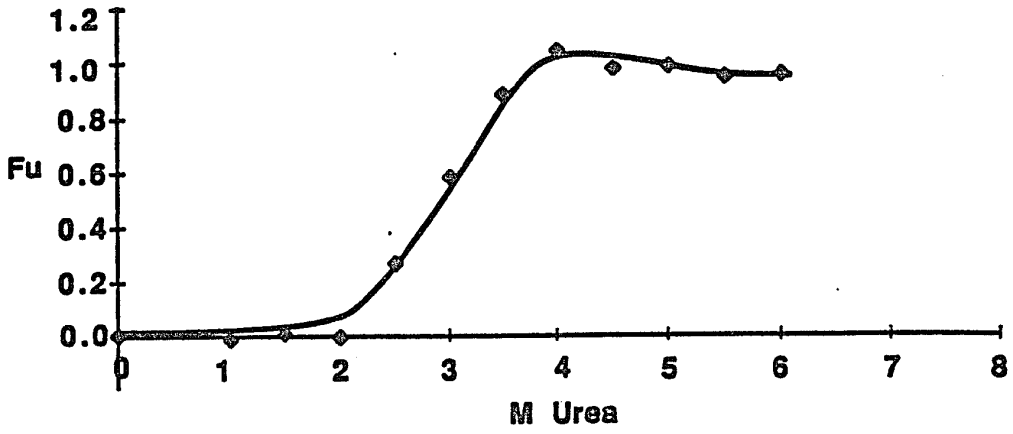

Figure 13. Fraction of unfolded RNase as a function of urea concentration at pH 4.0 and 17 ±°C.

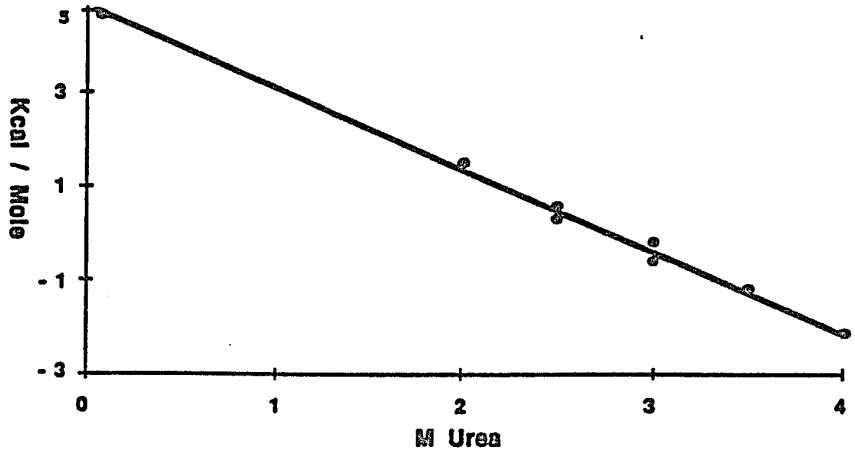

Figure 14. Change in Free energy of transition as a function of urea concentration at pH 4.0 and 17 ±°C.

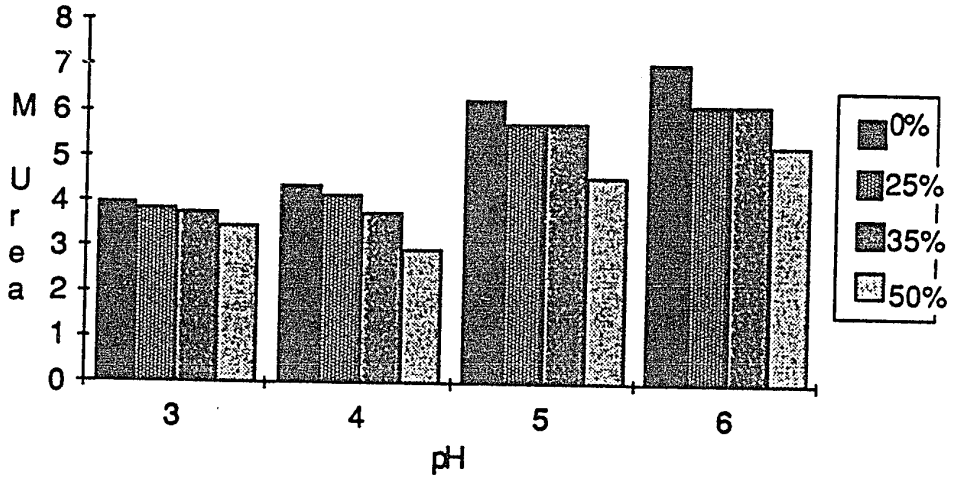

Figure 15. Cm as a function of pH at various methanol concentrations.

are independent of methanol concentration through 35% at pH 3, 4, and 5. At pH 6, a decrease in both the Cm and ΔG was observed with an increase in the methanol concentration (figure 16).

3.2.2 Effect of pH on absorbance during the urea-induced unfolding transition:

At any given concentration of methanol an overall increase in the Cm was observed as the pH was increased from 3 to 6 (figure 17). Under aqueous conditions, a small decrease in Cm was observed as the pH was increased from 3 to 4; this was followed by an increase in Cm as the pH was increased from 4 to 6.

This difference was found to decrease as the methanol concentration was increased. From this it can be seen that the protein is more stable at high pH and that the stability of the protein decreases with an increase in methanol concentration. The stability of protein at high pH is due to the charged-group effect which is due to the changes in the charges of the titratable side groups as the pH increases.

The free energy change was found to be small and

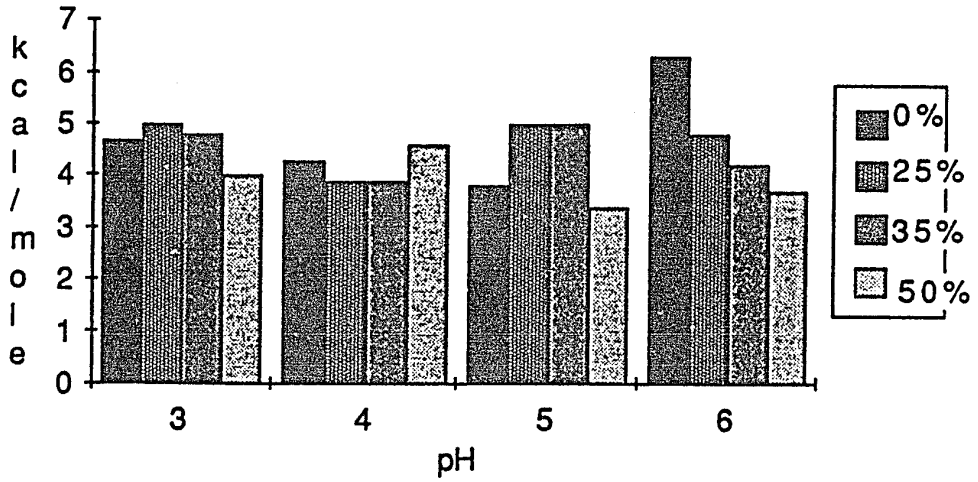

Figure 16. Change in free energy of transition as a function of pH at various methanol concentrations.

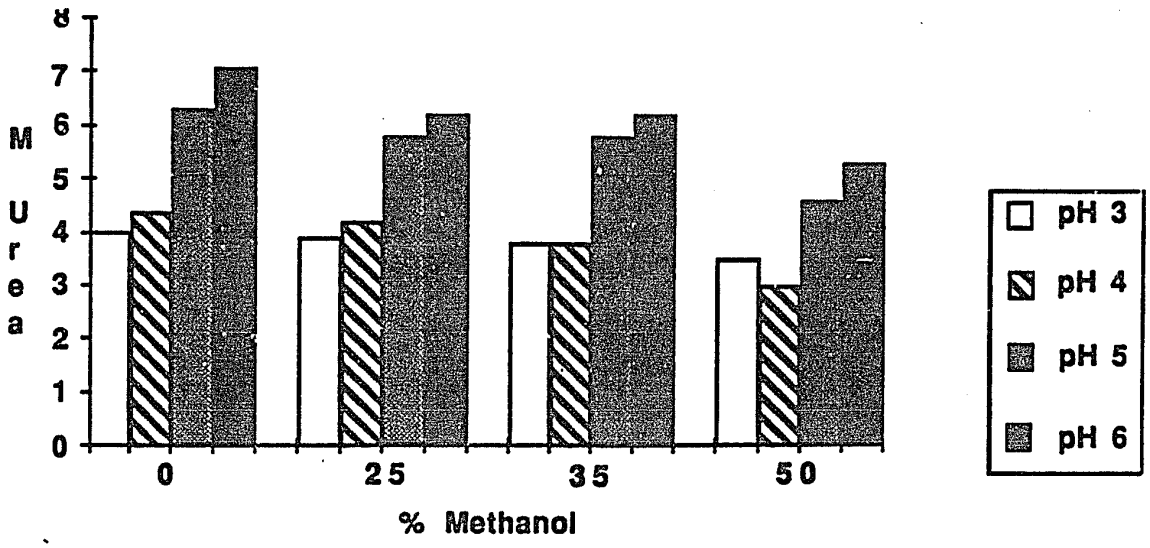

Figure 17. Cm as a function of methanol concentrations at pH 3, 4, 5 and 6.

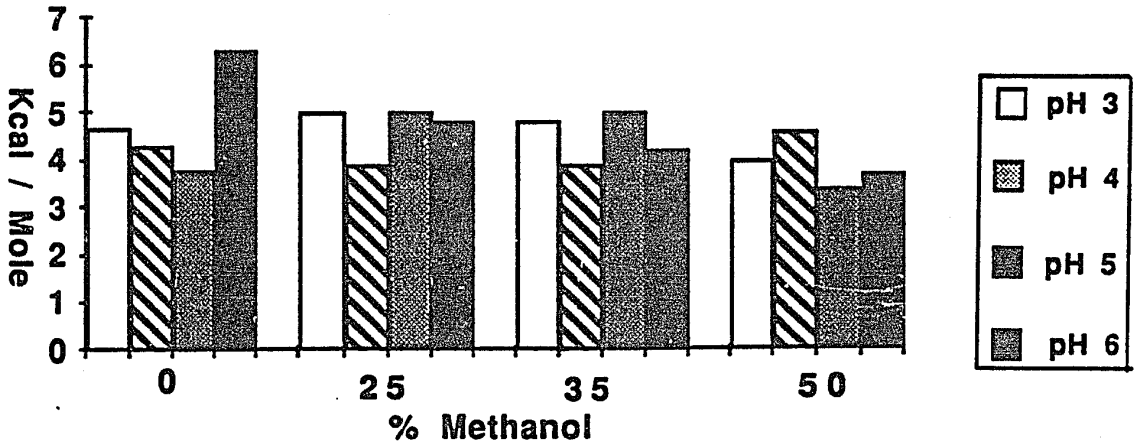

Figure 18. Change in free energy of transition as a function of methanol concentration at pH 3, 4, 5 and 6.

variable with increase in the pH (figure 18). This indicates that the native and the denatured states are stabilized to the same degree with increasing pH.

3.3 UREA-GRADIENT GEL ELECTROPHORESIS:

The urea-induced unfolding of RNase A was probed further with urea gradient gel electrophoresis to study the effect of change in pH and methanol concentrations on the stability of the protein. The urea-gradient gels for RNase A at a series of methanol concentrations at pH 3 are shown in Figure 19. When the mobilities of RNase A and carboxymethylated RNase A are compared, it is seen that while the mobility of RNase A shows a decrease with increasing urea concentration, the mobility of carboxymethylated RNase A is fairly linear, not affected by the urea concentration. When the Rf's were plotted as a function of urea concentration, a significant decrease in the mobility is observed between 5M and 6M urea (Figure 20), reflecting the unfolding of the protein in this range. Plots of the fraction of unfolded protein present and the change in free energy of transition as a function of urea concentration are shown in figure 21 and 22, respectively.

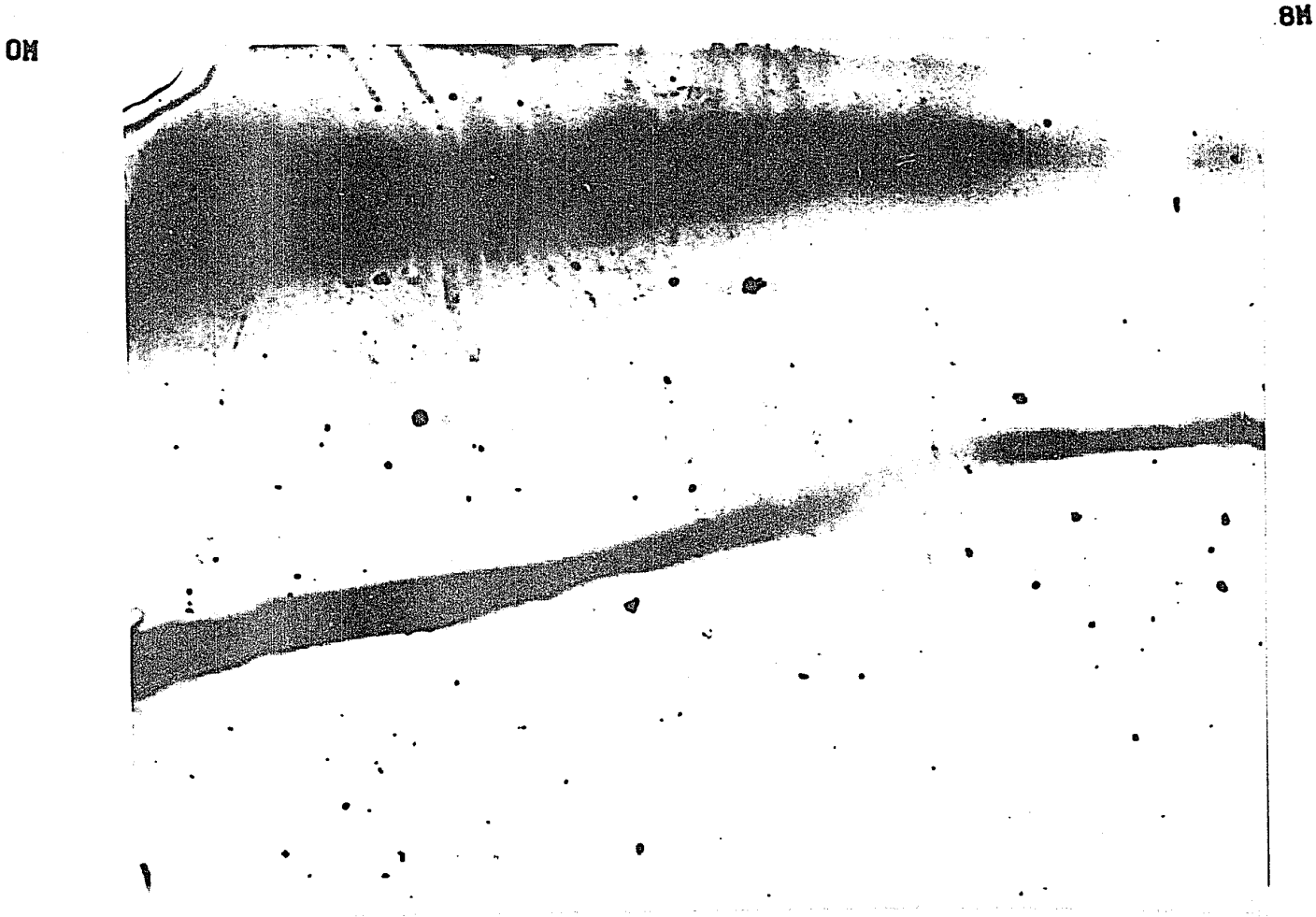

Figure 19a. Urea-Gradient Gel at pH 3.0, 0% methanol concentration electrophoresed at 17 ±°C.

Figure 19b. Urea-Gradient Gel at pH 3.0, at 17 ±^{0}C.

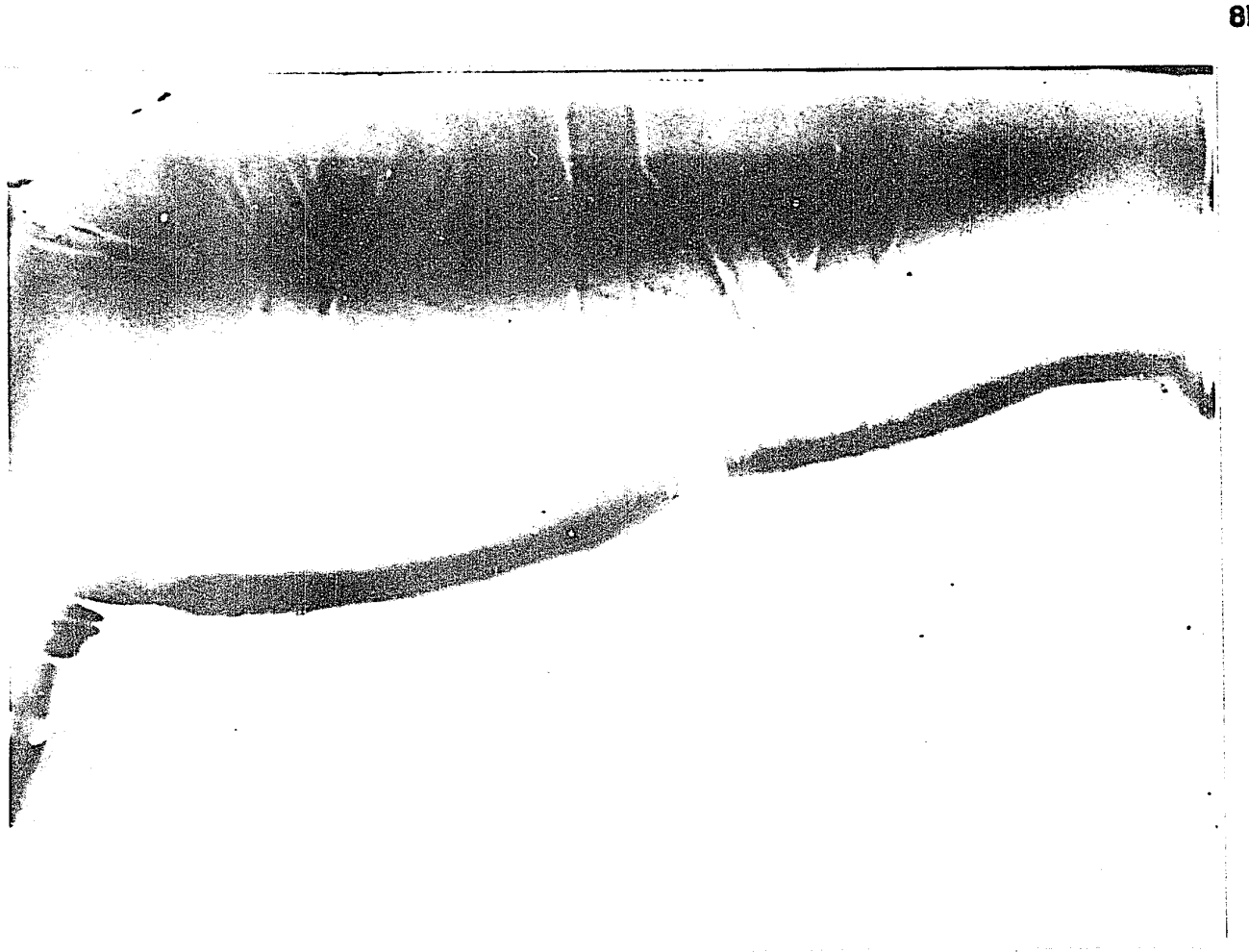

Figure 19c. Urea-Gradient Gel at pH 3.0 at 35% methanol concentration electrophoresed at 17 ± ^{0}C.

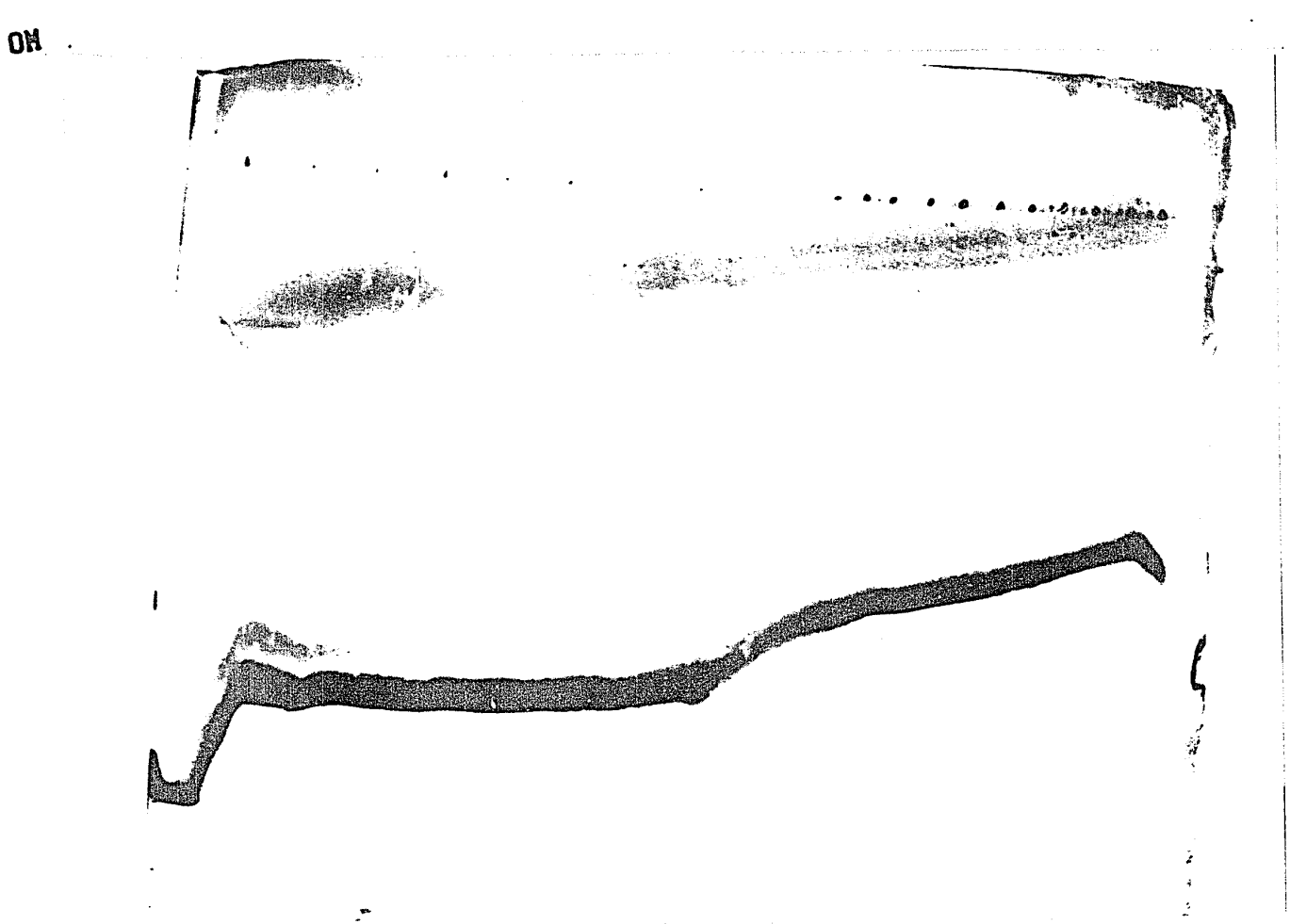

Figure 19d. Urea-Gradient Gel at pH 3.0, 50% methanol cocentration electrophoresed at 17 ± ^{0}C.

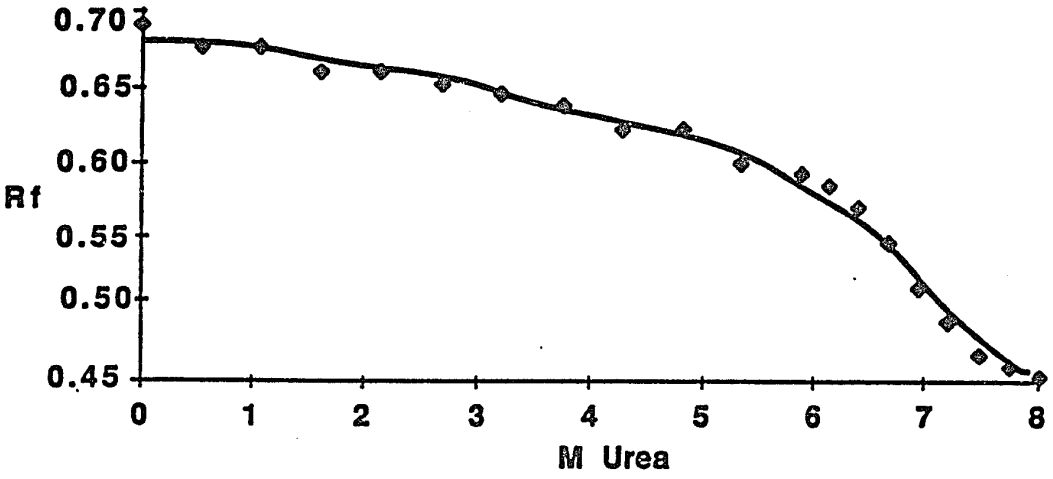

Figure 20. Relative mobility of RNase as a function of urea concentration at pH 3.0 and 17 ± ^{0}C.

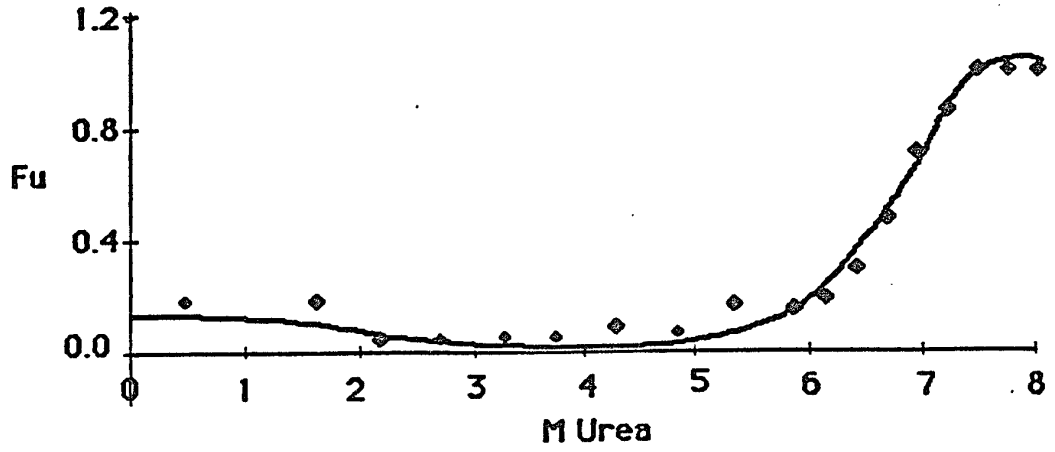

Figure 21. Fraction of unfolded RNase as a function of urea concentration at pH 3.0 and 17 ± ^{0}C.

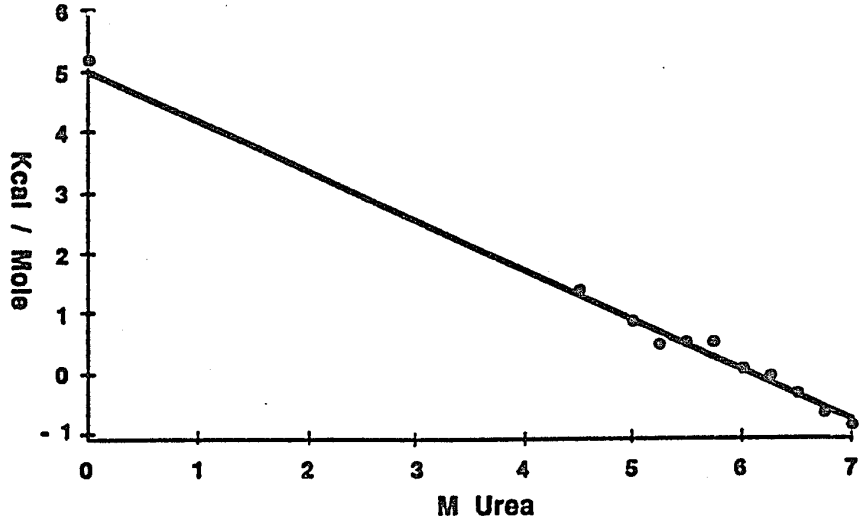

Figure 22. Change in free energy of transition as a function of urea concentration at pH 3.0 and 17 ± ^{0}C.

A decrease in the mobility of RNase A and carboxymethylated RNase A was observed with an increase in the pH. This is due to the change in the charge on the protein; i.e., the positive charge on the protein decreases when the pH increases thereby decreasing the mobility of the protein.

3.3.1 Effect of methanol on the urea-induced unfolding transition:

At pH 3 and 4, Cm was found to be independent of the methanol concentration up to 35% (figure 23) and decreases at 50%, whereas at pH 5, the Cm was found to be independent of the methanol concentration. The transition was found to become less cooperative, indicative of populated intermediates.

A marked decrease in the change in free energy was observed with an increase in the methanol concentration (figure 24). The changes in the transition free energies were found to be higher than those calculated from the absorbance and fluorescence data. The comparatively lower values obtained from the 35% and 50% urea-gel probably

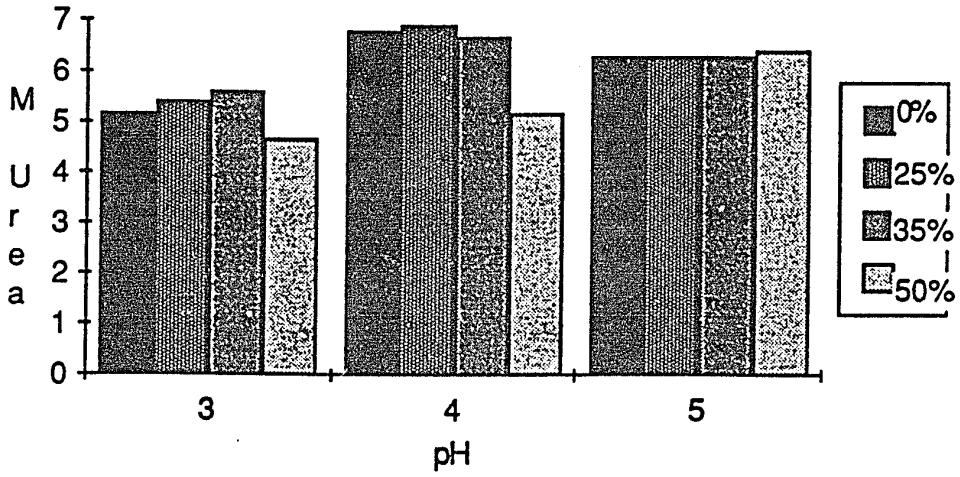

Figure 23. Cm as a function of pH at various methanol concentration.

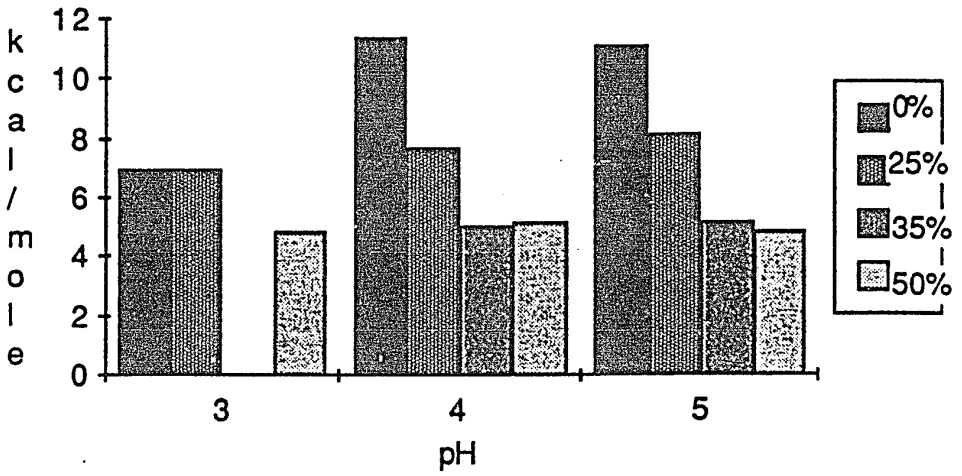

Figure 24. Change in free energy of transition as a function of pH at various methanol concentrations.

result from the fact that these values were obtained from data for which the transition was not completely cooperative. A bimodal transition was observed at pH 3 35% methanol condition. These values therefore represent an apparent free energy change for more than one transition, one of which has a low transition free energy.

3.3.2 Effect of pH on the urea-induced unfolding transition:

The urea-gradient gel transition was found to be sensitive to pH; i.e. a decrease in Cm was observed with a decrease in pH from 4 to 3 (figure 25). At pH 4 and 5, the Cm was found to be similar at all methanol concentrations. The free energy change was found to be variable with an increase in the pH at any given concentration of methanol (figure 26). The occurrence of a bimodal transition did not allow the analysis of ΔG for pH 3 35% methanol urea-gradient gels.

3.4 COMPARISON OF DATA OBTAINED FROM SPECTROSCOPIC PROBES AND UREA-GRADIENT GEL ELECTROPHORESIS:

When the data obtained from absorbance and

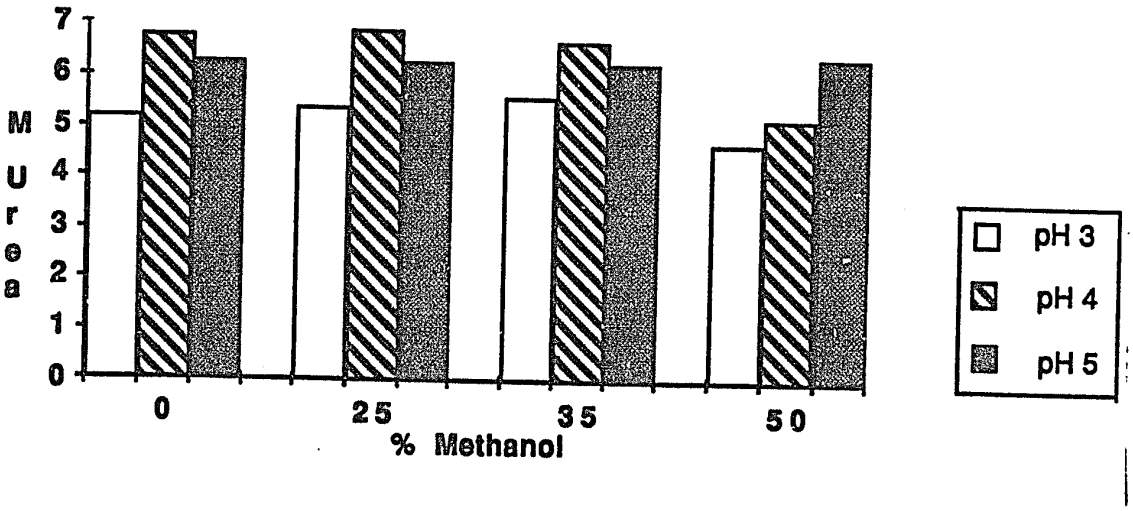

Figure 25. Cm as a function of methanol concentration at pH 3, 4 and 5.

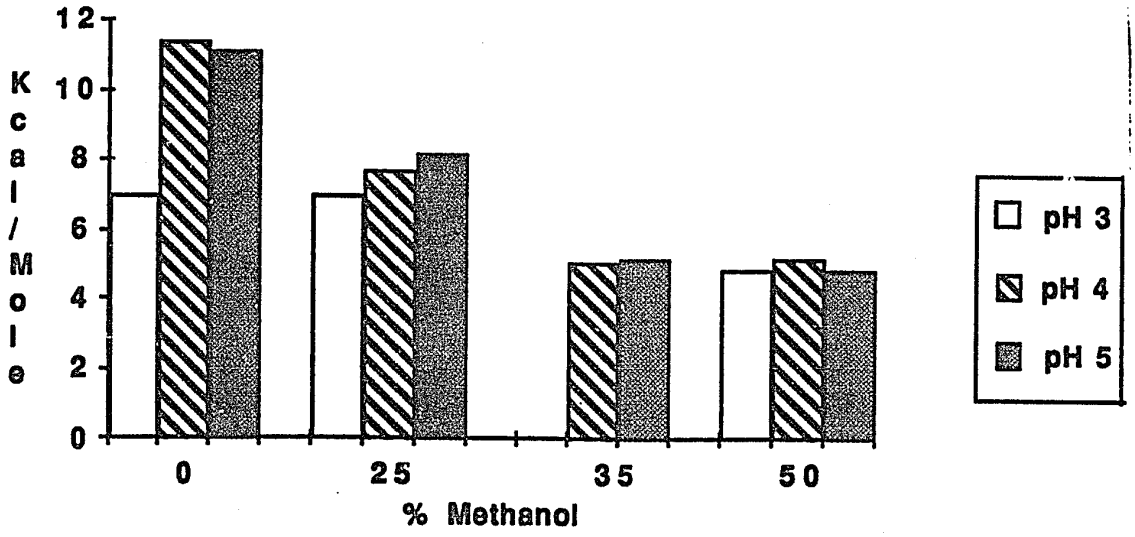

Figure 26. Change in free energy of transition as a function of methanol concentration at pH 3, 4 and 5.

fluorescence were compared, they were found to be non-coincident under a number of conditions. Only at pH 3, were the absorbance and fluorescence found to be coincident at all methanol concentrations. At pH 4, the absorbance and fluorescence were non-coincident under aqueous and 25% methanol conditions with absorbance having the lower Cm (figure 27), but were coincident with further increase in methanol concentration. Such non-coincidence is indicative of the possible presence of an intermediate. This also suggests that the two techniques monitor different events in the unfolding process. Absorbance monitors the formation of the intermediate from the native, whereas fluorescence monitors its decomposition. This intermediate which occurs at low urea concentration has the same absorbance as that of the unfolded state and fluorescence as that of the native state.

It was observed that under most of the conditions the data obtained from the spectrophotometric probes was not coincident with the urea-gel data. This is indicative of the presence of a second intermediate. Its formation is monitored by fluorescence and its decomposition by

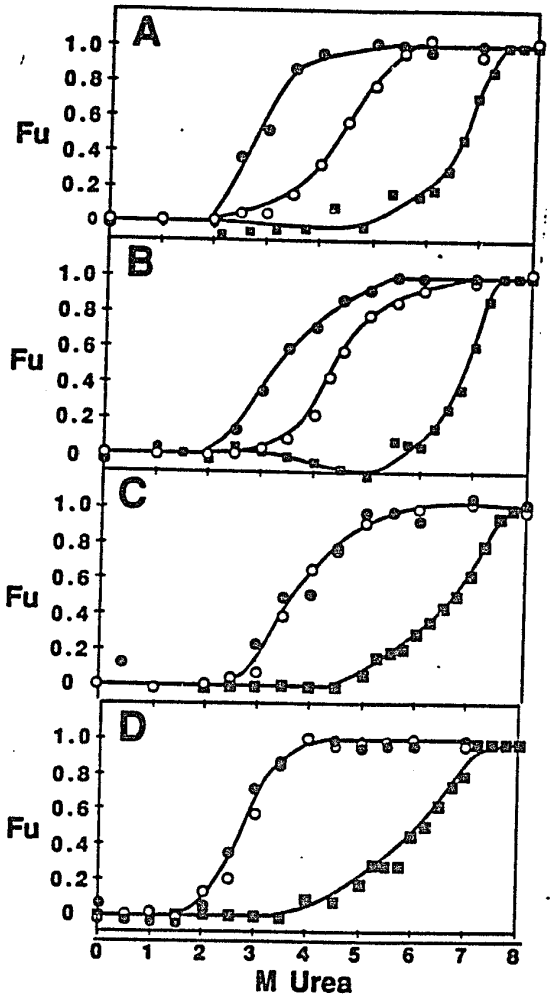

Figure 27. Comparison of the fraction of unfolded RNase data obtained from each technique. The filled circles represent absorbance data, the open circles represent fluorescence data and the filled squares represent the urea-gradient gel data. A) Aqueous; B) 25% (v/v) methanol; C) 35% (v/v) methanol; D) 50% (v/v) methanol.

urea-gradient gel electrophoresis. From the above interpretation, one can propose a scheme for the unfolding process as:

$$N \longrightarrow I_1 \longrightarrow I_2 \longrightarrow U.$$

The cooperativity of the transition seen from the gel data decreases with an increase in the methanol concentration (figure 28). At high methanol concentrations, it is seen that the two state transition no longer exists but instead a multi-state transition is present. In fact, the 35% data generally shows a two step unfolding transition. From this, the presence of a third intermediate or multiple intermediates can be suggested. These intermediates are partially unfolded as they have mobilities between that of the native and the unfolded state.

Therefore, from the above argument one can suggest this modified pathway for the unfolding of the protein:

$$N \longrightarrow I_1 \longrightarrow I_2 \longrightarrow I_2' \longrightarrow U.$$

When the data from the spectroscopic probes was

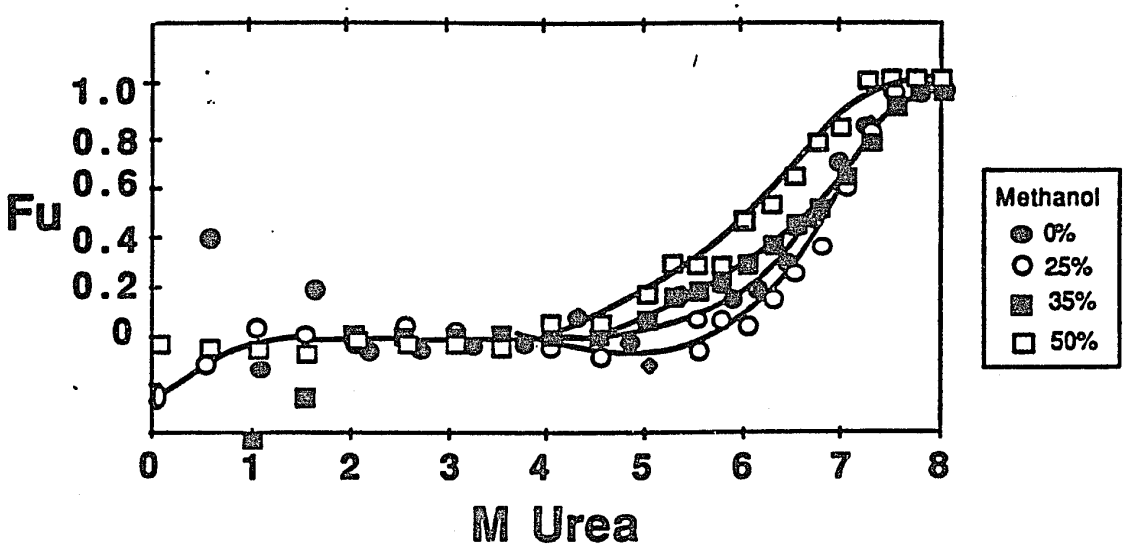

Figure 28. Fraction of unfolded RNase obtained from thr urea-gradient gel data as a function of urea concentration for various methanol concentration at pH 4.0 (Tris/acetate) and 17 ± ^{0}C.

compared to the gel data it was found that the intermediates seen during the absorbance and the fluorescence transition are fairly compact as their mobilities are similar to that of the native protein.

3.5 CHARACTERIZATION OF THE INTERMEDIATES:

3.5.1 Effect of pH on the stability of the intermediates:

Absorbance monitors the transition from N to I_1. From the absorbance data it was seen that with an increase in the pH, the Cm increases, whereas the free energy change was found to be fairly constant. This is consistent with the fact that the N and I_1 are being stabilized with increasing pH to the same extent.

The transition from I_1 to I_2 was monitored by the fluorescence. According to this data, an increase in the pH causes the Cm and free energy change to increase. From this it can be inferred that I_1 is stabilized with respect to I_2.

The transition from I_2 to U was monitored by urea-gel. An increase in the Cm and free energy was observed with an increase in the pH (from 3 to 4), whereas both remain fairly constant at higher pH. This indicates

that at low pH I_2 is stabilized with respect to U with increasing pH. At higher pH there is no effect.

3.5.2 Effect of methanol on the stability intermediates:

With the exception of pH 5 aqueous condition, both ΔG and Cm were found to be unaffected by methanol up to a concentration of 35%.

At pH 6 under all methanol concentrations and at higher methanol concentrations at all other pH's a decrease in the Cm and free energy change was observed. This is indicative of the destabilization of the native protein to a greater extent than I_1. It also shows that methanol destabilizes the native state, thus causing the transition to move to lower concentrations of urea as the methanol concentration increases. This effect stems from the more hydrophobic nature of the cosolvent as compared to the aqueous solution. Timasheff and coworkers (34 & 35) have shown that a major effect of several organic solvents on protein is the preferential exclusion of the cosolvent from the vicinity of the protein; that is, the protein is preferentially hydrated.

It is believed that the general effect of monohydric

alcohols, the preferential exclusion of cosolvents, reflects the unfavourable interaction between the relatively non-polar cosolvent and the charged surface of the protein (22). The more hydrophobic cosolvent is also expected to bind to the unfolded state with its greater hydrophobic surface area, as well as to partially-folded intermediate states with exposed non-polar surfaces.

The fluorescence data indicated the destabilization of I_1 by methanol as there was a decrease in Cm with increase in the methanol concentration. Based on the urea-gel data, it was found that the stability of I_2 was independent of the methanol concentration, whereas U and I_2' were stabilized by an increase in the methanol concentration.

3.6 COMPARISON OF UREA-INDUCED TRANSITION WITH OTHER DENATURANTS-INDUCED TRANSITION:

There have been a number of previous investigations on the effect of alcohols on the stability of the proteins. These include studies by Schrier et al. (36), Fink and Painter (37) and Gerlsma and Sturr (38) on RNase A and

other small monomeric proteins. Most of these studies involved the thermal unfolding of proteins and report a decrease in the transition temperature for unfolding in the presence of alcohols.

This is similar to the decrease noted in the Cm with an increase in the methanol concentration in case of urea-induced unfolding. The absorbance and the fluorescence data were found to be coincident in case of thermal unfolding of RNase A (37). Similar results were also observed in case of guanidinium hydrochloride unfolding. But, for the urea-induced unfolding transition the absorbance and fluorescence data were found to be non-coincident. This non-coincidence indicates that the intermediates which were not observed in case of thermal and guanidinium hydrochloride induced unfolding transitions are stable in urea solution. This is probably due to the fact that urea is a weaker denaturant than guanidinium hydrochloride according to the Hofmiester series (39). Therefore, the use of a weaker denaturant than urea may help populate many more intermediates.

The intrinsic absorbance and fluorescence of

proteins are frequently used as probes of folding. It is generally assumed that the protein is fully unfolded when the aromatic side-chains are fully exposed to solvent, i.e. when the change in either signal ceases to change. This may therefore be an erroneous assumption. Also in case of urea-gradient gel electrphoresis it was shown that the partially-folded intermediates observed in case of absorbance and fluorescence are quite compact. Preliminary circular dichroism results (Biringer, unpublished) have shown that the transitions observed by urea-gels and circular dichroism are non-coincident.

CHAPTER IV

CONCLUSION

The stabilizing effect of methanol cosolvents on the urea-induced unfolding transition of Ribonuclease A was studied by absorbance, fluorescence and urea-gradient gel electrophoresis under different solvent systems. The solvent conditions used were aqueous and aqueous-methanol solvents at pH 3, 4, 5 and 6.

An increase in the methanol concentration resulted in a decrease in the Cm. The transitions were found to be less cooperative and covered a wider range of urea concentrations. An increase in the methanol concentration affected fluorescence in a markedly different manner than absorbance, which strongly suggests that the two techniques serve to monitor different events in the unfolding process. The non-coincidence of the absorbance and fluorescence data for most of the conditions supports this hypothesis and indicates the presence of an intermediate which has absorbance similar to that of the unfolded state, but fluorescence similar to that of the native state.

The transition mid-points observed in the urea-gradient electrophoresis experiments were found to occur at higher concentrations of urea than the events monitored by the spectroscopic techniques.

This indicates that the partially-folded states monitored by absorbance and fluorescence are quite compact. The non-coincidence of the gel data with the absorbance and the fluorescence data suggests the presence of a second intermediate which has fluorescence similar to that of the unfolded state but is fairly compact. An increase in the methanol concentration showed the presence of a multi-state transition rather than the previously observed two state transition at aqueous and low methanol concentrations. This indicates the presence of a third partially-unfolded intermediate.

The transition free-energies obtained from absorbance and fluorescence were found to be similar but lower than those calculated fron the corresponding urea-gel experiments. These results were consistent with the gel-monitored transition representing the major unfolding event. The comparitively lower values obatined from the 35% and 50% urea-gels were probably due to the fact that these

values were obtained from data for which the transition was not completely cooperative. The values, therefore, represent an apparent free energy for more than one transition, one of which has a low transition free energy.

The model proposed on the basis of the above conclusions is as follows:

$$N \longrightarrow I_1 \longrightarrow I_2 \longrightarrow I_2' \longrightarrow U$$

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